ANTHRAQUINONES FROM THE ASPERGILLUS GLAUCUS GROUP ISOLATION, IDENTIFICATION AND BIOLOGICAL ACTIVITY

DISSERTATION

zur Erlangung des Grades eines Doktors

der Naturwissenschaften

der Fakultät für Biologie

der Eberhard-Karls-Universität Tübingen

vorgelegt von

ELHAM KOLTHOUM

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Abbreviations:

ATCC	American Type Culture Collection, Rockville, Md., USA
ATP	adenosine triphosphate
CBS	Centraalbureau voor Schimmelcultures, Baarn, the Netherland
cmp	counts per minute
CTP	cytidine triphosphate
DNA	deoxyribonucleic acid
ECA	Ehrlich ascites carcinoma
ETH	culture collection of Eidgenössischen Technischen Hochschule, Zürich, Switzerland
GTP	guanosine triphosphate
MIC	minimal inhibitory concentration
NCTC	Natural Collection of Type Cultures, London, UK
NJH	National Jewish Hospital and Research Centre, Denver, Coll., USA
NRRL	Northern Utilization Research and Development Division, Peoria, Ill., USA
PTLC	preparative thin-layer chromatography
PPO	2,5-diphenyloxazol
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rpm	revolutions per minute
TCA	trichloroacetic acid
TLC	thin-layer chromatography
Tris	tris (hydroxymethyl) aminomethane
Tü	Institut für Biologie II der Universität Tübingen, Lehrbereich Mikrobiologie
UTP	uridine triphosphate
v/v	volume per volume

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I. INTRODUCTION

Members of the *Aspergillus glaucus* group are widely distributed in nature. Most strains can be isolated from foodstuffs (Blaser, 1974; Chollet and Moreau, 1967) and are therefore of ecological importance. They were also described to be significant in the incipient spoilage of many organic materials characterized by high osmotic tension (Raper and Fennell, 1965) such as confectionery products (Speck, 1976) and condensed milk (Frazier and Westhoff, 1978). In addition, strains of the *A. glaucus* group are able to germinate and grow at minimum moisture levels, thus playing a considerable role in the spoilage of stored seeds and grain. As a consequence of their growth additional water is released and the invasion of other molds is facilitated (Semeniuk, Nagel and Gilman, 1947). Many species of this group were reported to grow on corn (Barron and Lichtwardt, 1959), on wheat (Carter and Young, 1950), on soybeans (Milner and Geddes, 1946) and on barley (Tuite and Christensen, 1952) stored at moisture levels of or above 14 percent. Members of the *A. glaucus* group were also encountered among fungi isolated from deteriorated textiles (Nopitsch, 1950).

Secondary metabolites of this group have been studied by many workers and a variety of compounds belonging to different chemical groups were isolated and identified. Gould and Raistrick (1934) isolated three crystalline pigments, flavoglaucin, auroglaucin and rubroglaucin, from the dried

mold substance of 25 species of the *A. glaucus* series. In addition to the above mentioned pigments, physcion was isolated from *A. glaucus* Link by Raistrick et al. (1937). In 1939, Ashley and coworkers reported that rubroglaucin was a mixture of two anthraquinones, physcion and the new erythroglaucin. Moreover, the presence of physcion anthranols A and B was reported. Recently, physcion, erythroglaucin and an anthranol of physcion were found to be produced by *A. ruber* (Richardson, 1976). The compounds catenarin, emodin, cynodontin and helminthosporin were reported to be possibly present. Catenarin and endocrocin were found by Shibata and Natori (1953) among the metabolic products of *A. amstelodami*. Echinulin, an indole metabolite which was previously isolated from *A. echinulatus* (Quilico and Panizzi, 1943), was also obtained. A related indole metabolite, neoechinulin, has been isolated as orange-red crystals from cultures of *A. amstelodami* (Barbetta et al., 1969; Casnati et al., 1973). Two years later, neoechinulins A and D have been reported to occure in *A. ruber* by Nagasawa et al. (1975). The latter compound was found to inhibit the growth of silk worm. In the same year, the occurrence of neoechinulin D in *A. amstelodami* was reported by Dossena and others. Cryptoechinulin A, yellowish crystals, was isolated from *A. amstelodami* during the investigation on echinulin biosynthesis (Cardillo et al., 1974). In 1976, more indole metabolites were isolated and identified. Hamasaki and coworkers (1976 a&b) obtained two new derivatives of L-alanyl-L-tryptophan anhydride from *A. chevalieri* and

three isoechinulins A, B and C were found by Nagasawa et al. (1976) in *A. ruber*.

Although the metabolites of the *A. glaucus* group have been chemically intensively investigated, their biological activity received relatively little attention. Few reports about antibiotic production by members of this group are available in the literature. In 1954, an antibiotic produced by *A. ruber* and *A. repens* was reported by Saito and Sameshima. Production of penicillin by *A. glaucus* species was noted by Blinc and Johanides (1956). A metabolite of *A. proliferans* able to inhibit the growth of tubercle bacillus was demonstrated by Gupta and Viswanathan (1955). The production of at least two antibiotics, amodin A and B, by a strain of *A. amstelodami* was reported by Darling et al. (1963). Two pimarane diterpenes, one having antibacterial activity and the other antiviral activity, were isolated by Ellestad and coworkers (1972) from *A. chevalieri*. Very recently, the production of cladosporin (identical to asperentin) by three species of the genus *Eurotium* was reported by Anke et al. (1978). Cladosporin inhibited the growth of several fungi and at very low concentrations the growth of *Bacillus brevis* and *Clostridium pasteurianum*. In the same year, asperentin and other metabolites were isolated by Podojil et al. from *E. repens* and their biological activity was investigated.

The above mentioned aspects make it interesting to investigate the biological activity of the pigements produced by

the *A. glaucus* group and to search for new secondary metabolites among the members of this group. This work deals with the isolation, purification, identification and characterization of four known and two new anthraquinone pigments as well as the study of their antimicrobial activity.

II. MATERIALS AND METHODS

A. MATERIALS

The reagent grade chemicals of E. Merck AG, Darmstadt, were used unless otherwise stated.

1. Chemicals for culture media

Bacto-agar	Difco, Detroit, Mich., USA
Bacto-nutrient broth	Difco, Detroit, Mich., USA
Malt extract	Oxoid Ltd., London, UK
Yeast extract	Oxoid Ltd., London, UK
D-Glucose	Deutsche Maizenawerke, Hamburg
Niax polyol LHT 67	Brenntag, Mülheim
Tween 80	Serva Feinbiochemica GmbH & Co., Heidelberg

2. Chemicals for biological tests

Gaunosine-5'-triphosphate (GTP)	Boehringer, Mannheim
Cytidine-5'-triphosphate (CTP)	Boehringer, Mannheim
Uridine-5'-triphosphate (UTP)	Boehringer, Mannheim
Thymidine, uracil and leucine	Boehringer, Mannheim
Calf thymus DNA	Boehringer, Mannheim
DNA-dependent RNA polymerase from *E. coli*	Boehringer, Mannheim
(2-^{14}C)-Thymidine 61 mCi/mmole	Radiochemical centre, Amersham, UK
(2-^{14}C)-Uracil 62 mCi/mmole	Radiochemical centre, Amersham, UK

(U-^{14}C)-Leucine 59 mCi/mmole	Radiochemical centre, Amersham, UK
(2-^{14}C)-Uridine 53.5 mCi/mmole	Radiochemical centre, Amersham, UK
(8-^{14}C)-Adenosine-5'-triphosphate 48 mCi/mmole	Radiochemical centre, Amersham, UK
Mice bearing Ehrlich ascites carcinoma (ECA)	were provided by Dr. H.G. Probst, Tübingen
Rifampicin	Ciba AG, Basel, Switzerland
Heparin	Serva Feinbiochemica GmbH & Co., Heidelberg

3. Anthraquinone derivatives

1,4,6,8,-Tetrahydroxy-anthraquinone	was a gift from Bayer AG, Leverkusen
Emodin	Fluka, Buchs, Switzerland
Other anthraquinone derivatives	were provided by Dr. H. Laatsch, Göttingen

4. Materials for chromatography

TLC aluminium sheets silica gel 60F_{254} pre-coated. Layer thickness 0.2 mm	E. Merck AG, Darmstadt
TLC aluminium sheets silica gel 60/kieselguhr F_{254} pre-coated. Layer thickness 0.2 mm	E. Merck AG, Darmstadt
PTLC plates silica gel 60 pre-coated. Layer thickness 2 mm	E. Merck AG, Darmstadt
Silica gel 60 for column chromatography, particle size 0.063 - 0.200 mm	E. Merck AG, Darmstadt

5. Microorganisms

For routine tests

Bacillus brevis Migula - ATCC 9999

Bacillus subtilis Cohn - ATCC 6633

Streptomyces viridochromogenes (Kriansky) Waksman et Henrici-Tü 57 (= ETH 23575)

For spectrum of antimicrobial activity

Arthrobacter citreus Sacks - ATCC 11624

Bacillus subtilis Cohn - ATCC 6051 and Tü 203

Escherichia coli (Migula) Castellani et Chalmers - K12

Proteus vulgaris Hauser - NCTC 4157

Pseudomonas fluorescens Migula - ATCC 13525

Sarcina lutea Schroeter - ATCC 381

Staphylococcus aureus Rosenbach - Tü 202 (= ETH 2070)

Streptococcus faecalis F.M. Harold - NJH 9790

Nocardia brasiliensis (Lindenberg) Pinoy - NRRL 2962

Streptomyces griseus Waksman et Henrici - Tü 6 (= ETH 7419)

Streptomyces antibioticus Waksman et Henrici - Tü 4 (= ETH 6677)

For pigment production

The following strains of the *Aspergillus glaucus* group (genus *Eurotium*) were kindly provided by Dr. P. Blaser, Zürich. ZT numbers were given to strains which were either isolated by him or of the ETH collection. Details about their origin are available in the dissertation "Taxonomische und physiologische Untersuchungen über die Gattung Eurotium Link ex Fries", P. Blaser, ETH Zürich, 1974.

E. acutum sp. nov. CBS 464.65 (ZT 8291)

E. acutum sp. nov. ZT 8356

E. amstelodami Mangin CBS 519.65 (ZT 8292)

E. appendiculatum sp. nov. ZT 8365

E. carnoyi Malloch et Cain CBS 471.65 (ZT 8294)

E. chevalieri Mangin ZT 8271

E. cristatum (Raper et Fennell) Malloch et Cain CBS 123.53 (ZT 8295)

E. echinulatum Delacr. ZT 8285

E. echinulatum Delacr. CBS 524.65 (ZT 8296)

E. echinulatum Delacr. ZT 8393

E. glabrum sp. nov. ZT 8218

E. herbariorum Link ex Fries ZT 8241

E. heterocaryoticum Christensen, Lopez et Benjamin CBS 411.65 (ZT 8299)

E. intermedium sp. nov. CBS 523.65 (ZT 8302)

E. leucocarpum Hadlok et Stolk CBS 516.65 (ZT 8300)

E. montevidensis (Talice et Mackinnon) Malloch et Cain CBS 491.65 (ZT 8293)

E. niveo-glaucum (Thom et Raper) Malloch et Cain CBS 517.65 (ZT 8304)

E. pseudoglaucum (Blochwitz) Malloch et Cain CBS 123.28 (ZT 8305)

E. repens De Bary ZT 8362

E. rubrum König, Spieckermann et Bremer CBS 530.65 (ZT 8301)

E. spiculosum sp. nov. ZT 8275

E. spiculosum sp. nov. ZT 8277

E. spiculosum sp. nov. ZT 8279

E. tonophilum Ohtsuki ZT 8242

E. Umbrosum (Bain et Sartory) Malloch et Cain CBS 117.46 (ZT 8308)

6. Culture media

The following liquid media were used (content per liter).

For bacteria

Nutrient broth 8.0 g, pH 7.2.

For actinomycetales

Yeast extract 4.0 g, malt extract 10.0 g and D-glucose 4.0 g, pH 7.2.

Synthetic medium for *Bacillus brevis* (modified after Eikhom et al., 1963)

K_2HPO_4 13.6 g, $(NH_4)_2SO_4$ 2.0 g, $CaCl_2$ 10.0 mg, $MgSO_4 \cdot 7\ H_2O$ 0.2 g, $FeSO_4 \cdot 7\ H_2O$ 0.5 mg, L(+)-glutamic acid 5.0 g and bidistilled water to 1l, pH 7.2.

Media for evaluation of the pigment production

1: Malt extract 20.0 g, pH 5.2.

2: Malt extract 20.0 g and NaCl 100.0 g, pH 5.0.

3: Malt extract 20.0 g, NaCl 50.0 g and D-glucose 100.0 g, pH 5.2.

4: Malt extract 15.0 g, NaCl 50.0 g, D-glucose 100.0 g and yeast extract 5.0 g, pH 5.3.

5: Malt extract 20.0 g and NaCl 25.0 g, pH 5.1.

6: Malt extract 20.0 g, NaCl 25.0 g and yeast extract 0.5 g, pH 5.3.

7: Malt extract 20.0 g, NaCl 25.0 g and yeast extract 5.0 g, pH 5.3.

N.B.: Distilled water was used for all media. For preparation of solid medium, 20 g agar per liter liquid medium were added.

B. METHODS

1. Cultivation and extraction

E. cristatum was maintained on agar slants of malt extract medium. The spore suspension from one slant served to inoculate two agar plates (14 cm Ø) containing the same medium. The plates were incubated for 2 - 4 weeks at room temperature until they were well sporulated. The spores were suspended in 50 ml tween solution (one drop tween in 50 ml water).

Submerged cultures of the organism were grown in 500 ml Erlenmeyer flasks with one buffle containing 150 ml of medium. The flasks were inoculated each with 2 ml spore suspension and incubated at 27^{o} C on a rotatory shaker (120 rpm) for two weeks.

The cultures were filtered under vacuum and the mycelia were left to dry in the dark for few hours at room temperature. The culture filtrate was extracted with an equal volume of ethyl acetate, while the mycelia were extracted with acetone (50 ml/g). The obtained organic extracts were concentrated at 30^{o} C under vacuum to 5 % of their original volumes. Aliquots were used for test of antibiotic activity and for TLC.

Effect of different cultural conditions on pigment-production

The influence of the incubation temperature (23^{o}-37^{o} C), the starting pH (4.0-6.5) and the constitution of the me-

dium (media 1,2,4-7) on the production of the pigments by *E. cristatum* was studied by comparing the antimicrobial activity of the obtained organic extracts as well as their chromatographic behavior.

2. Fermentation

The fermentation conditions were:

- 10 l (type MF 14 New Brunswick, Scientific Co., New Brunswick, USA): 180 rpm, 3 l air/min, at 28° C.
- 20 l (type b 20, B. Braun Melsungen AG, Melsungen) with "intensor" system: 800 rpm, 6 l air/min, at 28° C.

The required volume of medium No. 5 (pH 5.1) was inoculated with 50 or 100 ml spore suspension. An alcoholic polyol solution was used as antifoam agent when needed. The antimicrobial activity of the culture and the pH of the culture broth were regularly estimated during the period of fermentation (two weeks).

3. Test of antibiotic activity

The antibiotic activity of the culture broth and the mycelia was routinely tested by the plate-diffusion method following the procedure described by Zähner and Maas (1972).

Preparation of a spore suspension of *B. brevis* and *B. subtilis* (Klein, 1957; Wolf, 1971)

An agar plate (14 cm Ø) was inoculated with 2 ml of an 18-hours old bacterial culture and incubated for 12 days at

37° C. The culture was then suspended in 50 ml water, heated in a water bath for 30 min at 80° C and centrifuged (UJ III, Heraeus Christ GmbH, Osterode/Harz) at 3000 rpm for 10 min. After decantation, the sediment was resuspended in 50 ml water. The steps of heating, centrifugation, decantation and resuspension in water were repeated three times and the obtained suspension was kept at 3° C.

Preparation of a spore suspension of *Streptomyces* species

A diluted yeast extract medium (1:1 with water) was used. A culture of the organism on agar plate (14 cm Ø) was incubated for 8-10 days at 27° C. The produced spores were then suspended in 50 ml of sterile tween solution, shaken vigorously and kept at 3° C.

Preparation of the plates

The required volume of sterile agar was distributed in 17.5 ml-portions into sterile plastic Petri dishes having an internal diameter of 85 mm (C.A. Greiner and Söhne, Nürtingen). Five-ml inoculated agar (10^6 bacterial spores/ml) were poured on top of the solidified base layer.

In the case of *Actinomycetales*, plates containing a single layer of 10 ml solid seed medium (10^6 spores/ml) were prepared.

Plating

Filter paper discs (6 mm diameter, MN 827, Macherey, Nagel and Co., Düren) were impregnated with the test solution and applied to the agar surface. The filter discs were either

dipped in the culture broth and dried on a filter paper (MN 804, Macherey, Nagel and Co., Düren) or pipetted with 10 µl of the concentrated organic extract and left to dry on a glass plate. After an incubation period of 18-24 hours at 27° C or 37° C, according to the test organism, the inhibition zones were measured.

4. Bioautography

Bioactive spots were detected by means of bioautography as described by Betina (1975). Measured volumes of the organic extracts of the mycelia and of the culture filtrates were applied onto TLC silica gel sheets and developed in chloroform-methanol (97:3 v/v). Strips of the dried chromatographic sheets were cut and placed on the surface of inoculated agar plates. The plates were incubated at the appropriate temperature for 16-24 hours and the inhibition zones formed arround the strips were measured.

5. Isolation and purification of the pigments

E. cristatum was grown on medium No. 5 at 27° C for two weeks. The mycelium, containing considerably larger amounts of pigments than the culture filtrate, was extracted several times with acetone. The acetone extracts were combined, concentrated to a smaller volume, and dried under vacuum at 30° C. The residue was dissolved in chloroform and filtered if necessary. The chloroform solution was applied onto a silica gel column (4.6 x 50 cm). Elution was carried

out with chloroform followed by 3 %, 5 % and 10 % methanol in chloroform. Fractions containing the same pigments were detected with the aid of TLC in chloroform-methanol (97:3 v/v) and combined. The following pigments were isolated:

Fraction I: This fraction contained a yellow pigment and some colourless impurities. The colourless impurities were removed by PTLC on silica gel in chloroform-methanol (97:3 v/v). The yellow band was carefully scratched, packed in a small column and eluted with chloroform-methanol (90:10 v/v). Further purification was achieved by crystallization from ethyl acetate giving long orange-yellow needles of pigment I (physcion).

Fraction II: It was composed of one orange and two yellow pigments; one of the yellow pigments was identical to pigment I. The three components of this fraction were separated by PTLC on silica gel in carbon tetrachloride-chloroform (90:10 v/v). The chromatograms were developed twice in the freshly prepared solvent system and the bands were treated as mentioned above. The orange pigment II (erythroglaucin) was crystallized from chloroform-methanol (90:10 v/v) at 3^{o} C giving small dark red needles. The third pigment II_{a} (physcion anthrone) was obtained by evaporation of the solvent leaving a pale yellow powder which was further purified by recrystallization from the same solvent.

Fraction III: This fraction was orange-red with green fluorescence. It was found to contain a bright red pigment, the above mentioned mixture, as well as traces of another yel-

low pigment. The amount of this yellow pigment was too small to be characterized. In order to obtain the red pigment, the solvent was evaporated and the residue was extracted several times with petroleum benzine (60^{o}-80^{o} C) to get rid of the orange mixture. Finally the remaining undissolved part was dissolved in a small volume of chloroform and left to crystallize in the refrigerator. The obtained crystals, which were orange-red plates, were filtered, washed and recrystallized from the same solvent giving dark red needles of pigment III (catenarin).

Fraction IV: This fraction was composed of three main pigments; pigment III, a violet (IV) and a red substance (IV_a). These substances were separated from each other by PTLC using benzene-ethyl acetate-acetic acid (45:55:1 v/v) as developing solvent system. The bands were eluted as mentioned under "Fraction I" and catenarin, the violet and the red pigments (IV: viocristin and IV_a: rubrocristin) were then crystallized from chloroform, ethyl acetate and ethanol respectively.

6. Identification

Thin-layer chromatography

The identity and the purity of the pigments at different steps of the isolation were checked by TLC. The formed spots were studied directly in day light and in ultraviolet light at 365 nm. The R_f values of the isolated substances were determined using silica gel or silica gel kieselguhr as sorbents. Silica gel plates were developed in

chloroform-methanol (97:3 v/v), carbon tetrachloride-chloroform (90:10 v/v) or in benzene-ethyl acetate-acetic acid (45:55:1 v/v) while silica gel-kieselguhr plates were developed in petroleum benzine (40^{o}-60^{o} C)-ethyl formate-formic acid (90:4:1 v/v).

Ultraviolet-visible spectra

A self recording Beckman DB-GT spectrophotometer was used to obtain the visible and ultraviolet absorption spectra of pigments III and IV_a in absolute ethanol. Runs were repeated using ethanol plus one drop of 1 N NaOH as solvent.

Infrared spectra

IR spectra of pigments III and IV_a were determined by KBr disc technique with a Perkin Elmer Model 21 spectrophotometer. The disc contained 200 mg KBr and 0.5 mg of the pigment.

7. Pigment production in other strains of the *A. glaucus* group

The metabolites produced by twenty five strains of the *A. glaucus* group in different media (1-4) were extracted as described for *E. cristatum*. The pigments were identified by TLC in four solvent systems using those isolated from *E. cristatum* as references.

8. Biological activity of catenarin and other related compounds

a. Agar plate diffusion test

The test was performed as previously described and the antimicrobial activity of the pigments against different microorganisms was investigated. Three filter paper discs were used for each test solution (0.5 mg/ml in ethanol) and the average of the inhibition zones was taken.

b. Serial dilution test

The minimal inhibitory concentration (MIC) of the substances under investigation against *B. brevis* and that of catenarin against a variety of microorganisms were estimated. The arithmetic-series type of dilution test described by Kavanagh (1963) was used. Volumes of a 0.5 mg/ml solution ranging from 1-25 µl in increments of 1 or 2 µl, were pipetted into test tubes containing 0.5 ml of the inoculated liquid medium. After incubation, the tubes were visually inspected and the lowest concentration which inhibited growth was taken as the MIC.

c. Photometric assay

The effect of catenarin and 1,4,6,8-tetrahydroxyanthraquinone upon growth of *B. brevis* in liquid medium was studied turbidimetrically. The biophotometer (Jouan, Paris) described by Coultas and Hutchison (1962) and Poralla (1967) was used and the MIC of both compounds in complex and synthetic media were determined. A volume of medium was ino-

culated with 2 ml of an overnight culture, grown in the assay medium, and incubated at 37° C on a rotatory shaker (120 rpm) until an optical density of 0.1-0.11 at 578 nm (Eppendorf photometer 110M) was reached. The cuvettes, each containing 7.5 ml of this growing bacterial culture, were placed in the incubation chamber at 37° C and maximum amplitude and frequency of the magnetic stirrer. The pigment, dissolved in 0.1 ml ethanol, was added at the indicated time and the same volume of ethanol was added to the control.

d. Bactericidal activity of catenarin

A growing culture of *B. brevis* was prepared as mentioned under photometric assay. A sample was taken ($t_o$count) just before the addition of the test solution and the culture was then incubated at 37° C on a rotatory shaker. Viable-cell counts were made at time intervals by plating 0.05 ml volumes of suitably diluted aliquots of the culture on well-dried agar, incubating as usual and then counting the resulting colonies (modified after Miles and Misra, 1938).

e. Effect on macromolecular syntheses in *B. brevis*

The macromolecular syntheses by intact cells of *B. brevis* in presence of some polyhydroxyanthraquinones was investigated according to the method described by Siddhikol et al. (1969) and Anke (1979). The organism was grown in nutrient broth to an optical density of 0.5 at 578 nm. The test substance was added followed after 5 min with (U-^{14}C) leucine, (2-^{14}C) uracil or (2-^{14}C) thymidine each 0.05 µCi per ml

and the culture was reincubated for 10 min at 37° C with gentle shaking. Aliquots were mixed with an equal volume of cold 10 % trichloroacetic acid (TCA) containing unlabeled precursor at 1 mM. The TCA-insoluble material was collected on membrane filters (0.45 µm pore size, 25 mm Ø; Sartorius, Göttingen), washed twice with 5 % TCA and dried. The filters were placed in vials with 5 ml scintillation fluid (4 g PPO/l toluene) and the radioactivity was determined in a Mark II liquid scintillation counter (Mark II, Model 8660, Nuclear Chicago, Des Plaines, Ill., USA).

f. Effect on macromolecular syntheses in ECA cells

The influence of the pigments on the incorporation of leucine, uridine and thymidine into TCA-insoluble material in ECA cells was studied following the procedure described by Anke et al. (1977). The cells (3×10^6) suspended in phosphate buffered saline containing heparin were added to the compound under test and incubated for 10 min at 37° C. 2 ml volumes were then transferred to test tubes containing 0.1 µCi (2-^{14}C) uridine, 0.1 µCi (U-^{14}C) leucine or 0.1 µCi (2-^{14}C) thymidine and incubated for 20 min at 37° C. After centrifugation at 0° C, the pellet was suspended in 5 % TCA and the acid-insoluble material was treated as mentioned above.

g. Effect on DNA-dependent RNA polymerase from *E. coli* (EC 2.7.7.6)

The procedure described by Zillig et al. (1966) and Fuchs et al. (1967) was followed.

The following reagents were used per test (0.1 ml): T4 DNA, 20 µg/ml; ammonium chloride, 0.13 M; magnesium acetate, 10 mM; UTP, CTP and GTP each at 1 mM; (8-^{14}C) ATP, 0.5 mM; phosphoenolpyruvate, 10 mM; pyruvate kinase, 20 µg/ml; Tris acetate pH 7.9, 0.03 M; RNA polymerase 36 µg (18 units)/ml.

After incubation at 37° C for 20 min, the reaction mixture was diluted with 0.5 ml of ice-cold water and precipitated by the addition of 0.7 ml of ice-cold 10 % TCA. The TCA-insoluble material was collected on membrane filters and the radioactivity was determined as described before.

h. Binding to DNA

The interaction between some anthraquinone derivatives and DNA was followed by spectroscopy in the visible range applying the method described by Swanbeck (1966). The absorption spectrum of the pigment solution in phosphate buffer ($0.75.\ 10^{-3}$M Na_2HPO_4 and $0.25.10^{-3}$M NaH_2PO_4, pH 7.2) was compared with that of a similar solution containing DNA. DNA was dissolved in buffer (100 mg/l) and a small volume of ethanol containing the substance under test was then added to give a concentration of 4 mg/l. The absorption spectra (against blanks containing no pigment) were measured in a Zeiss PM QII spectrophotometer. In the case of physcion and erythroglaucin, cuvettes having 5 cm path length were used. The effect of DNA on the spectrum of emodin solution was studied in presence and absence of magnesium ions ($MgCl_2$: emodin molar ratio of 10/1).

III. RESULTS

1. Production of pigments under varying cultural conditions

The incubation temperature did not affect the pigment production, but the growth rate was slightly decreased when the temperature was increased (Table 1).

Table 1: Effect of incubation temperature on the growth of *Eurotium cristatum**.

Temperature	23° C	28° C	32° C	35° C
Mycelial weight g/l	15.7	15.1	14.3	14.0

* medium 5, starting pH 5.1

The starting pH of the medium had no influence at all on the yield of the pigment as long as the growth was not impaired. No differences were observed at pH values ranging from 4 - 5.5 while the growth rate of the culture was slightly lower above pH 6.0.

The constituents of the medium, especially glucose and salt concentrations, had a significant effect on both the growth and the production of pigments (Table 2). The yield of pigments per gram of mycelium was highest on medium 5, which contained 0.5 M sodium chloride. When the salt concentration was raised to 2.0 M (medium 2), the growth was slightly inhibited and smaller amounts of pigments were detected. The addition of yeast extract (medium 7) resulted in somewhat higher mycelia weight, however, the pigment production

Table 2: Effect of the constitution of the medium on growth and pigment production of *E. cristatum*.

Medium	Mycel	Physcion or " anthrone		Erythro-glaucin		Caten-arin		Rubro-cristin		Vio-cristin	
	g/l	my	cb	my	cb	my	cb	my	cb	my	cb
1	14.3	++	+	++	+	+++	+	++	+	++	++
2	8.5	++	+	++	-	++	+	+	-	+	-
4	28.8	++	+	++	+	+++	+	++	+	++	+
5	10.2	+++	+	+++	+	++++	+	++	+	++	+
6	8.8	++	+	++	+	++	+	+	+	++	+
7	12.5	+	+	+	-	-	-	-	-	-	-

my = mycelia

cb = culture broth

\+ = the number of + indicates the relative amounts of pigment produced per gram mycel on different media.

was drastically decreased. The presence of glucose (medium 4) had a marked effect on the growth. The highest mycelial weight was obtained on this medium but the content of pigment per gram was nearly the same compared to medium 1 or 6 and less compared to medium 5. The quantitative determination of the pigments of *E. cristatum* produced on three different media is given in Table 3. It is obvious from these data that the overall yield was highest on medium 4.

Table 3: Quantitative determination of the pigments produced by *E. cristatum* on different media.

Substance	mg/l of culture		
	Medium 5	Medium 4	Medium 7
Physcion-9-anthrone	4.2	12.5	-
Physcion	13.0	12.0	6.0
Erythroglaucin	45.0	42.0	4.0
Catenarin	57.5	70.0	-
Rubrocristin	4.0	5.0	-
Viocristin	6.0	5.0	-
Mycelial weight (g/l)	10.2	28.8	12.5

2. Fermentation

The production of biologically active pigments was followed by testing the antibacterial activity during fermentation. The results presented in Fig. 1 show that the activity, which was only detectable after 5-6 days of incubation, increased markedly up to the eleventh day. After this period, either a slightly higher or a lower activity of the mycelium or the culture broth was observed.

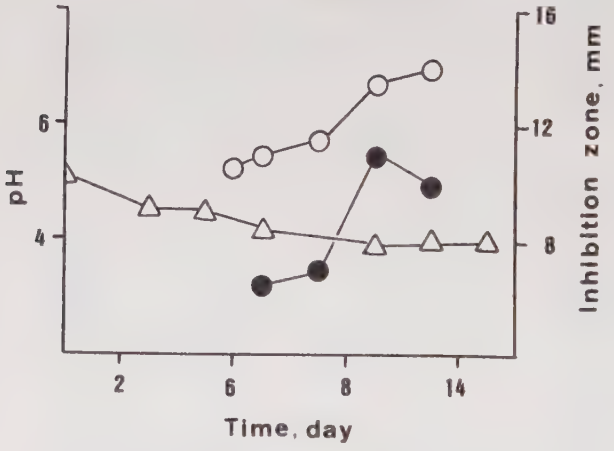


Fig. 1: Antibiotic production during fermentation of *E. cristatum* ○ - ○ , activity of the mycelium and ● - ● , activity of the culture broth against *B. brevis*; △ -△ , pH

3. Isolation and purification

Six crystalline pigments were obtained from the mycelium of *E. cristatum* by column chromatography and PTLC. The isola-

tion and purification were carried out as shown in Fig. 2. The main constituents of the pigment mixture were catenarin and erythroglaucin forming 44.3 % and 34.7 % of the total amount produced on medium 5. The content of physcion was 10 % while the other three components formed the remaining 11.0 %.

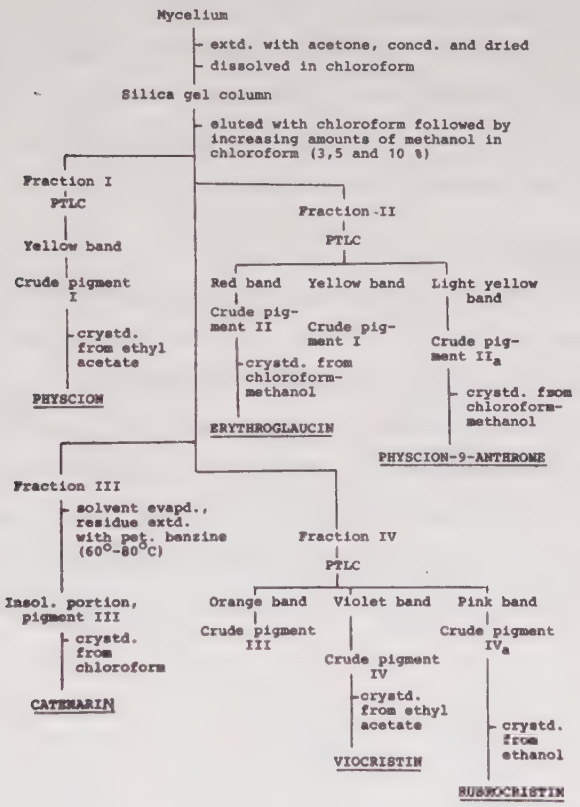


Fig. 2: Isolation and purification procedure of the pigments of *E. cristatum*

4. Physico-chemical characterization

General properties of the isolated pigments are summarized in Table 4. All the pigments are soluble in aqueous sodium hydroxide giving deep coloured solutions.
Melting points were determined by Dr. H. Laatsch, Göttingen.

Thin-layer chromatography

The R_f values and the methods of detection of emodin and the pigments isolated from *E. cristatum* are given in Table 5. Emodin was studied in order to investigate its production by other strains of the genus *Eurotium*.

UV-visible spectra

Fig. 3 and 4 show the ultraviolet-visible absorption spectra of catenarin and rubrocristin, the absorption maxima of which were at 232 and 230 nm. Other absorption bands were observed at 254 - 257, 280 and 300 - 303 nm. It is obvious from these results that the two pigments are closely related derivatives of the same parent molecule. In an alkaline solution, the spectra of both compounds exhibited a base shift, which is characteristic for phenolic chromophores.

Table 4: General properties of the isolated pigments.

Property	Physcion	Physcion-anthron	Erythro-glaucin	Catenarin	Vio-cristin	Rubro-cristin
Colour and shape	orange-yellow needles	pale yellow needles	deep red needles	brick-red needles	fein dark violet powder	dark red prisms
Melting point	204° C	260° C	206° C	245° C	decomp. above 300° C	decomp. above 300° C
Solubility in MeOH	partially sol.	almost insol.	slightly sol.	moderately sol.	partially sol.	slightly sol.
Solubility in EtOH	"	"	"	"	"	"
Solubility in chloroform	partially sol.	almost insol.	slightly sol.	moderately sol.	almost insol.	almost insol.
Solid + conc. H_2SO_4	ruby red	yellow → dark green	blue	violet	dark blue	blue → violet
Solid + 1 N NaOH	cherry-red	dissolves slowly → yellow soln.	violet	intense violet	violet → blue	violet
Alc. soln. + Na_2CO_3	-	-	-	red	violet	purple
Alc. soln. + $NaHCO_3$	pink	-	purple	pink → red	violet	purple

Table 5: R_f values of the isolated pigments.

Solvent system	Physcion	Physcion-anthrone	Erythro-glaucin	Caten-arin	Vio-cristin	Rubro-cristin	Emodin*
Benzene-ethyl acetate-acetic acid (45:55:1)	0.63	0.62	0.62	0.57	0.41	0.34	0.57
Chloroform-methanol (97/3)	0.70	0.70	0.71	0.44	0.39	0.36	0.46
Pet. benzine (40^{o} - 60^{o} C)-ethyl formate-formic acid (90:4:1)	0.22	0	0.29	0.02	0	0	0.02
Carbon tetrachloride-chloroform (90:10)	0.13	0.02	0.19	0	0	0	0
			Detection				
Day light	dark yellow	light yellow	light red	orange-red	violet	pink	orange-yellow
UV light	dark yellow	light red	orange	orange	purple	pink	yellow
Bioautogram	-	-	-	+	-	-	...

* Authentic emodin.

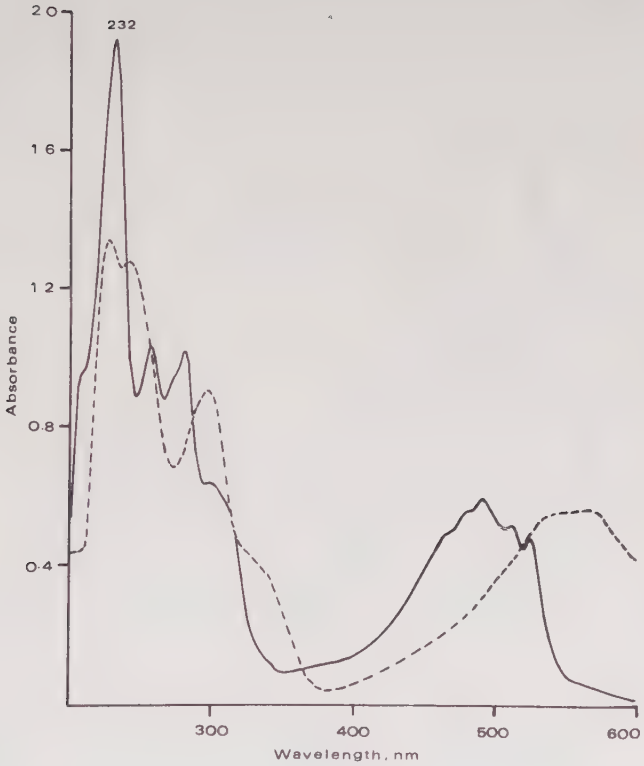


Fig. 3: UV-vis.-absorption spectra of catenarin

—— in ethanol, --- in ethanol + 1 drop 1 N NaOH

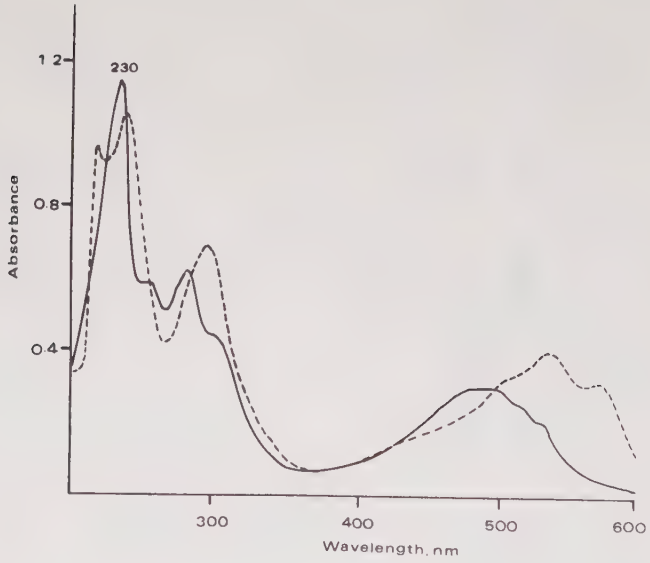


Fig. 4: UV-vis.-absorption spectra of rubrocristin

—— in ethanol, --- in ethanol + 1 drop 1 N NaOH

Infrared spectra

The infrared spectra of catenarin and rubrocristin are presented in Fig. 5 and 6. The former had a characteristic band at 1600 cm^{-1} (carbonyl) and that of the latter was at 1590 cm^{-1}. Rubrocristin had another absorption band at 2900 cm^{-1} (unchelated OH), which was not predominated in the spectrum of catenarin.

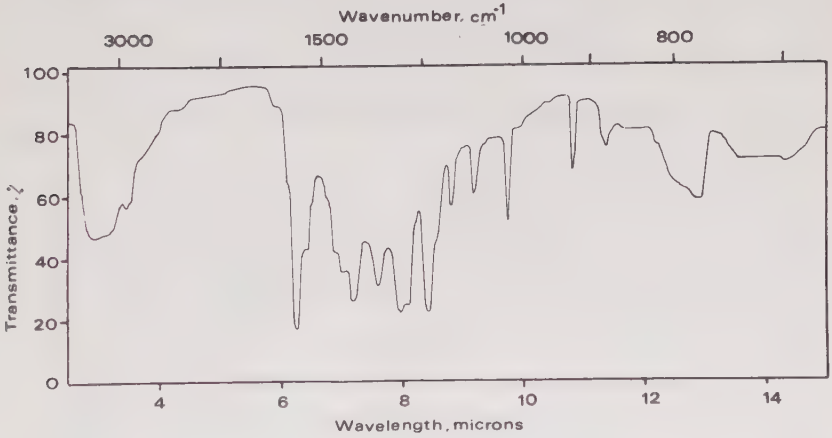


Fig. 5: Infrared absorption spectrum of catenarin.

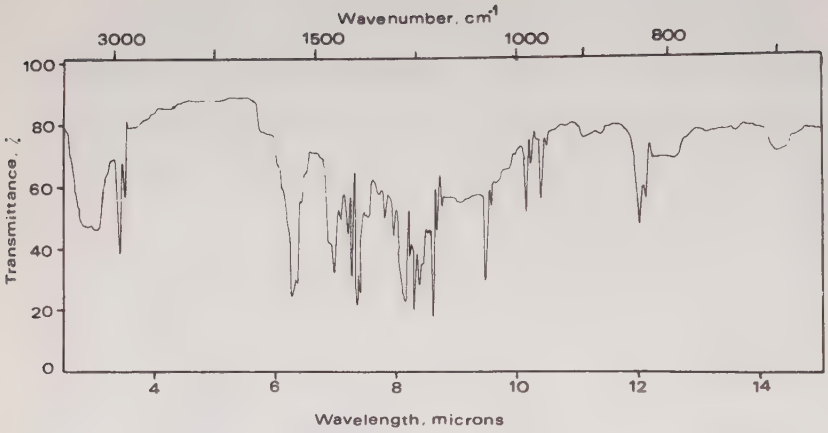
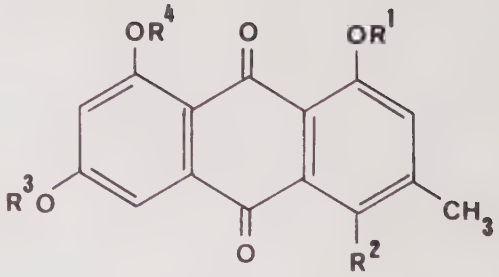
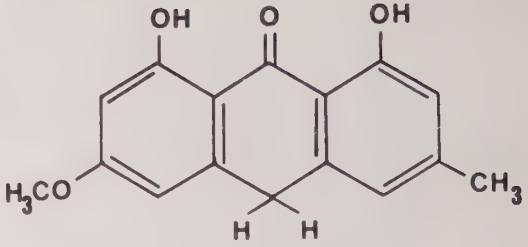


Fig. 6: Infrared absorption spectrum of rubrocristin.

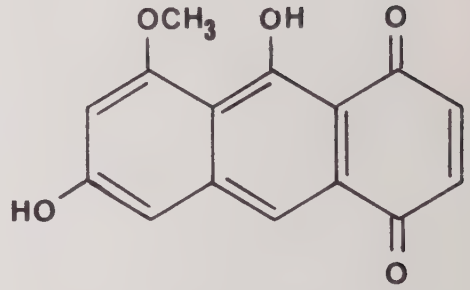
Further chemical identification and structure elucidation were done by Dr. H. Laatsch, Organisch-Chemisches Institut der Universität Göttingen. Details are given by Anke et al. (Arch. Microbiol., 1980). Structures of the isolated anthraquinones are shown in Fig. 7. Pigments IV and IV_a have not been described as natural products, therefore the names viocristin and rubrocristin were suggested.



	R^1	R^2	R^3	R^4
Physcion	H	H	Me	H
Erythroglaucin	H	OH	Me	H
Catenarin	H	OH	H	H
Rubrocristin	H	OH	H	Me



Physcion-9-anthrone



Viocristin

Fig. 7: Structures of the isolated anthraquinones

5. Production of pigments by other strains of the *A. glaucus* group

Many members of the *A. glaucus* group are capable of synthesizing all the pigments isolated from *E. cristatum* (Table 6). The investigated strains were classified into three subgroups according to Blaser (1974): *E. herbariorum*, *E. echinulatum* and *E. amstelodami*. The highest number of species producing all the pigments was under group III. In group I, pigment production was higher on medium 4, whereas all tested strains of *E. echinulatum* (group II) produced more pigments on medium 3. No rubrocristin or viocristin could be produced by species of group II and non of the pigments could be detected in cultures of *E. montevidensis*, *E. amstelodami* and *E. heterocaryoticum* (group III).

Generally, viocristin and rubrocristin were found to occure together with catenarin suggesting common biosynthetic pathways.

When *E. cristatum* is compared with the other members of the *A. glaucus* group, it can be noticed that this organism produced easily detectable amounts of all pigments on the simplest and cheapest medium (1). This favoured the selection of *E. cristatum* for the production of large amounts of pigments.

Table 6: Occurrence of anthraquinones in the mycelia (my) and culture broth (cb) of different strains of the genus *Eurotium*.

Strain and No.	Medium	Physcion or " anthrone		Erythro-glaucin		Catenarin		Rubrocristin		Viocristin		Emodin	
		my	cb	my	cb	my	cb	my	cb	my	cb	my	cb
Group I													
Eurotium acutum 8291	2	++	-	+	-	+	-	-	-	-	-	-	-
E. acutum 8291	4	++	-	++	-	++	-	-	-	-	-	-	-
E. acutum 8356	2	-	-	++	-	-	-	-	-	-	-	-	-
E. acutum 8356	4	++	+	++	+	+	+	-	-	-	-	-	-
E. glabrum 8218	3	+	+	++	+	++	+	++	+	++	+	-	-
E. herbariorum 8241	2	-	-	+	-	-	-	-	-	-	-	-	-
E. herbariorum 8241	4	++	-	++	-	-	-	-	-	-	-	-	-
E. pseudoglaucum 8305	2	-	-	-	-	-	-	-	-	-	-	-	-
E. pseudoglaucum 8305	4	+	-	+	-	-	-	-	-	-	-	-	-
E. repens 8362	3	++	+	++	-	++	-	-	-	-	-	-	-
E. repens 8362	4	++	+	++	+	+	-	-	-	-	-	-	-

Table 6. Contin.

E. rubrum 8301	2	+	+	+	-	+	-	-	-	-	-	-	-
E. rubrum 8301	4	++	+	++	+	++	+	+	-	++	-	+	-
E. tonophilum 8242	2,4	+	-	-	-	-	-	-	-	-	-	-	-
E. umbrosum 8308	3	++	+	++	+	++	+	++	+	++	+	-	-
Group II													
E. appendiculatum 8365	2	-	-	-	-	-	-	-	-	-	-	-	-
E. appendiculatum 8365	4	+	-	+	-	-	-	-	-	-	-	-	-
E. carnoyi 8294	2,4	+	-	+	-	-	-	-	-	-	-	-	-
E. echinulatum 8285	2	+	-	+	-	-	-	-	-	-	-	-	-
E. echinulatum 8285	4	-	-	-	-	-	-	-	-	-	-	-	-
E. echinulatum 8296	3	+	+	++	-	++	+	-	-	-	-	-	-
E. echinulatum 8296	4	++	+	+	-	-	-	-	-	-	-	-	-
E. echinulatum 8393	3	++	+	++	-	++	+	+	-	-	-	-	-
E. echinulatum 8393	4	+	+	+	-	+	-	-	-	-	-	-	-
E. niveoglaucum 8304	2	+	-	+	-	-	-	-	-	-	-	-	-
E. niveoglaucum 8304	4	+	-	++	-	+	-	-	-	-	-	-	-

Table 6. Contin.

Group III													
E. amstelodami 8292	2,4	-	-	-	-	-	-	-	-	-	-	-	-
E. chevalieri 8271	2	++	-	+	-	++	+	+	-	+	-	-	-
E. chevalieri 8271	4	++	+	++	-	++	+	++	+	++	+	-	-
E. cristatum 8295	1,4	++	+	++	+	++	+	++	+	++	+	+	+
E. heterocaryoticum 8299	3,4	-	-	-	-	-	-	-	-	-	-	-	-
E. intermedium 8302	2	-	-	+	-	-	-	-	-	-	-	-	-
E. intermedium 8302	4	+	-	+	-	-	-	-	-	-	-	-	-
E. leucocarpum 8300	3	-	-	+	-	-	-	-	-	-	-	-	-
E. leucocarpum 8300	4	++	+	++	+	+	-	-	-	-	-	-	-
E. montevidensis 8293	2,4	-	-	-	-	-	-	-	-	-	-	-	-
E. spiculosum 8275	3	++	+	++	+	++	+	++	+	++	+	-	-
E. spiculosum 8277	3	++	+	++	+	++	+	++	+	++	+	-	-
E. spiculosum 8279	1	++	+	++	+	++	+	++	+	++	+	-	-

++: easily detectable amounts were produced; +: only traces were produced

- : no detectable pigments were produced

6. Biological activity of catenarin and other related compounds

a. Antimicrobial spectra

Table 7 shows the antimicrobial spectra of the isolated pigments and a structurally related synthetic anthraquinone (1,4,6,8-tetrahydroxyanthraquinone). Catenarin was the most active compound among the metabolites of *E. cristatum*. Tetrahydroxyanthraquinone showed a slightly higher activity, viocristin and emodin were less active than catenarin, while rubrocristin, physcion and erythroglaucin had no inhibitory effects. The most sensitive organisms were *B. brevis*, *B. subtilis* and *Streptomyces viridochromgenes*. Gram negative bacteria were less sensitive, only *Proteus vulgaris* was affected. Yeasts and filamentous fungi were also tested; *Aspergillus cervinus* Tü 676, *A. stramenius* Tü 690, *Candida albicans*, *Dipodascus albidus*, *Endomycopsis fibuliger*, *Fusarium cubense*, *Hansenula anomala*, *Nadsonia fulvescens*, *Nematospora coryli*, *Neurospora crassa*, *Pichia farinosa*, *Rhodotorula rubra*, *Saccharomyces cerevisae* and *Schizosaccharomyces pombe* were all not sensitive at concentrations up to 20 µg/disc.

Raising the concentrations of the active substances up to 20 µg/disc did not increase the inhibition zones indicating poor diffusion in the agar medium.

Table 7: Antimicrobial spectra of 1,4,6,8-tetrahydroxyanthraquinone (I), catenarin (II), rubrocristin (III), emodin (IV), physcion-9-anthrone (V), physcion (VI), erythroglaucin (VII) and viocristin (VIII).

Organism	Temp.	Inhibition zone (mm/10 µg of the pigment)							
		I	II	III	IV	V	VI	VII	VIII
Arthrobacter citreus	A	9.0	8.0	-	7.0	-	-	-	-
Bacillus brevis	B	10.5	8.0	-	7.0	-	-	-	7.0
B. subtilis ATCC 6633	B	8.5	7.5	-	7.0	-	-	-	-
B. subtilis ATCC 6051	B	6.5	+	-	-	-	-	-	-
B. subtilis Tü 203	B	6.5	6.5	-	+	-	-	-	+
Escherichia coli K12	B	-	-	-	-	-	-	-	-
Nocardia brasiliensis	A	8.5	+	-	6.5	-	-	-	-
Proteus vulgaris	B	6.5	+	-	+	-	-	-	+
Pseudomonas fluorescens	A	-	-	-	-	-	-	-	-
Sarcina lutea	B	-	-	-	-	-	-	-	-
Staphylococcus aureus	B	+	-	-	-	-	-	-	-
Streptococcus faecalis	B	-	-	-	-	-	-	-	-
Streptomyces griseus	A	8.0	+	-	7.5	-	-	-	-
Streptomyces antibioticus	A	-	-	-	-	-	-	-	-
Streptomyces viridochromogenes	B	10.5	8.0	-	8.0	-	-	-	-

+: very small inhibition zone; -: no inhibition zone; A: 27° C; B: 37° C

Table 8: Antimicrobial spectrum of catenarin.
Inoculum size: 10^6 cells or spores/ml, medium: nutrient broth for bacteria and yeast extract medium for actinomycetes; method: serial dilution assay.

Organism	Incubation temperature	MIC µg/ml
Arthrobacter citreus	37° C	16.0
Bacillus brevis (S)	37° C	2.0
B. brevis	37° C	1.0
B. subtilis ATCC 6633 (S)	37° C	4.0
B. subtilis ATCC 6633	37° C	3.0
B. subtilis ATCC 6051	37° C	23.0
Proteus vulgaris	37° C	> 25.0
Staphylococcus aureus	37° C	19.0
Streptomyces antibioticus (S)	27° C	> 25.0
Streptomyces viridochromogenes (S)	37° C	18.0

(S): spores

In liquid medium, catenarin showed markedly higher inhibitory effects against many of the sensitive organisms (Table 8). The growth of *B. brevis* and *B. subtilis* from spores was inhibited at 2 and 4 µg/ml. *Staph. aureus* showed no or very low sensitivity to catenarin in the agar plate method, however its growth was inhibited at 19 µg/ml in the serial dilution test. In addition, the activity of tetrahydroxy-

anthraquinone against *B. brevis* was equal to that of catenarin as shown in Table 9. The MIC of both compounds was 1 µg/ml. Viocristin and emodin were also more active in liquid medium than in the agar diffusion assay, whereas rubrocristin, physcion and erythroglaucin caused no growth inhibition up to a concentration of 20 or 25 µg/ml. Higher concentrations could not be used due to the low solubility of these compounds.

Table 9: Antibacterial activity of the isolated pigments, emodin and 1,4,6,8-tetrahydroxyanthraquinone against *B. brevis* (10^6 cells/ml) in nutrient broth.

Substance	MIC µg/ml
Catenarin	1
Emodin	5
Erythroglaucin	> 20*
Rubrocristin	> 25
Physcion	> 20*
Tetrahydroxyanthraquinone	1
Viocristin	5-10

* the compound was not completely dissolved; after incubation, formation of some precipitate was observed.

b. Bactericidal activity of catenarin

The bactericidal activity of catenarin was investigated as mentioned in the experimental section and the resultant survival curves are presented in Fig. 8. During the first hour of contact, about 20 and 26 % of the cells ($1.28.10^7$ and $1.38.10^7$ cells/ml at t_o) were killed at 5 µg and 10 µg/ml. After that, a sudden increase of the bactericidal rate was observed at the higher concentration. In this case up to 50 % of the bacterial population was killed in the following 30 minutes and only 0.30 % could survive after 270 minutes.

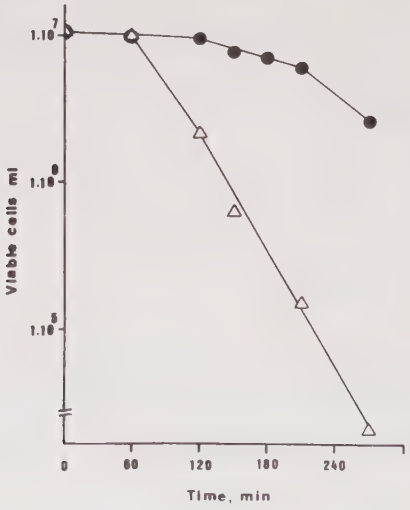


Fig. 8: Bactericidal activity of catenarin.

● - ● : 5 µg/ml, △ - △ : 10 µg/ml.

c. Photometric assay

The influence of catenarin and tetrahydroxyanthraquinone on growth of *B. brevis* in relation to the growth phase and nutrient medium is shown in Fig. 9-11. When catenarin was added at the beginning of the log phase (Fig. 9, A), 1 µg/ml prevented growth. When the same amount was added at a higher population density (B), the growth ceased for 6 hours and continued afterwards.

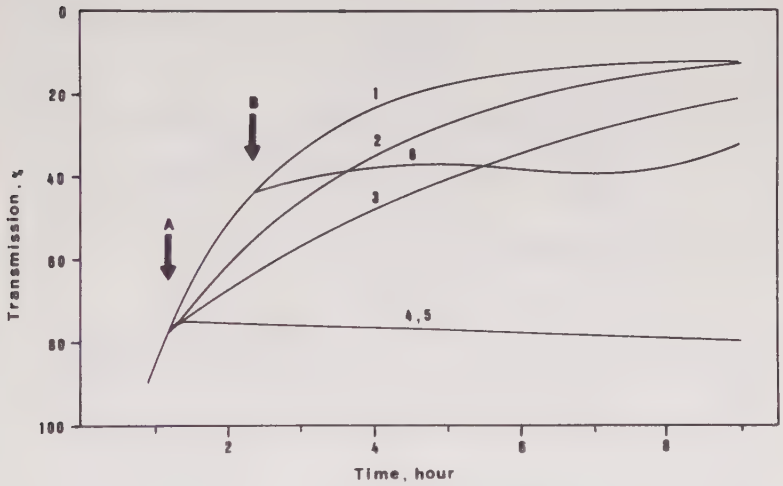


Fig. 9: Effect of catenarin on the growth of *B. brevis* in nutrient broth.
1: control 2: 0.5 µg/ml (A); 3: 0.75 µg/ml (A); 4: 1 µg/ml (A); 5: 2 µg/ml (A) 6: 1 µg/ml (B)
The arrows indicate the addition of the antibiotic.

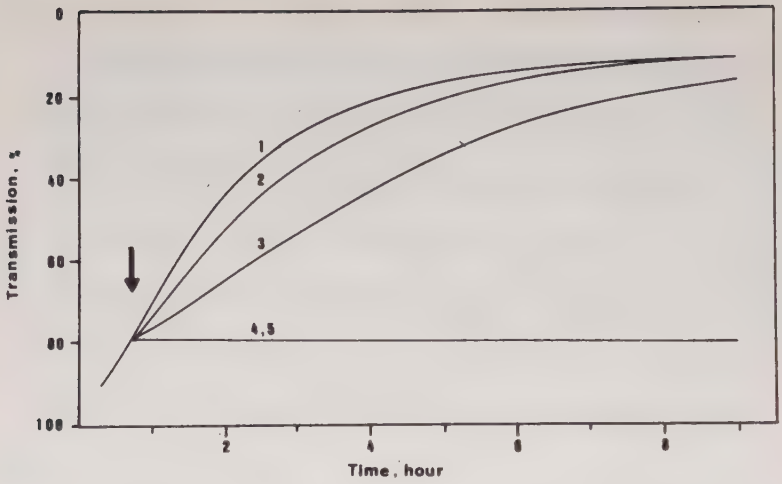


Fig. 10: Effect of 1,4,6,8-tetrahydroxyanthraquinone on the growth of *B. brevis* in nutrient broth.
1: control 2: 0.5 µg/ml 3: 0.75 µg/ml
4: 1.0 µg/ml 5: 2.0 µg/ml

The inhibitory effect of tetrahydroxyanthraquinone towards *B. brevis* was exactly the same as that of catenarin. Its MIC in complex medium was 1 µg/ml (Fig. 10), which is in agreement with the results of the serial dilution test. In synthetic medium, the activity of both anthraquinones was significantly higher inhibiting the growth at a five-fold lower concentration (Fig. 11).

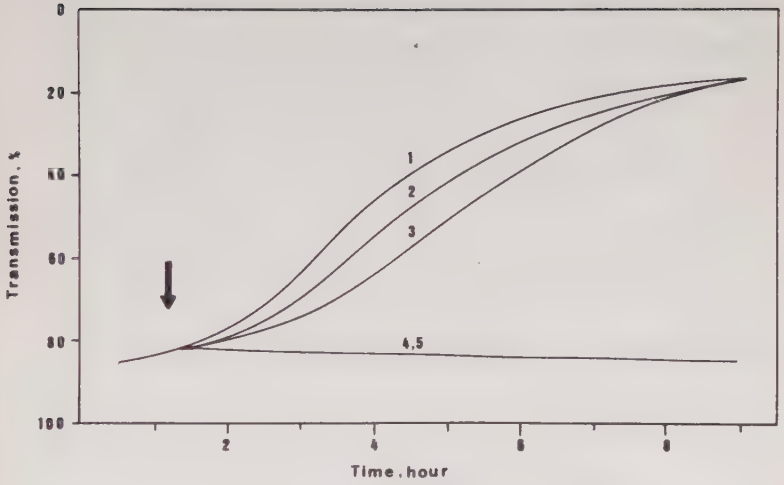


Fig. 11: Effect of catenarin and 1.4.6.8-tetrahydroxyanthraquinone (THA) on the growth of *B. brevis* in synthetic medium.

1: control
2: 0.1 µg/ml THA
3: 0.1 µg/ml catenarin
4: 0.2 µg/ml THA
5: 0.2 µg/ml catenarin

d. Inhibition of macromolecular syntheses in *B. brevis*

Fig. 12-14 show the effect of catenarin and rubrocristin on the incorporation of uracil, leucine and thymidine into TCA-insoluble fractions in growing cells of *B. brevis*. Catenarin inhibited the incorporation of uracil and leucine at concentrations as low as 0.1 µg/ml. Increasing the concentration to 1 µg/ml, their incorporation was almost completely inhibited. The incorporation of thymidine was affected to a lesser extent. Rubrocristin, having no growth inhibitory activity, had no considerable influence on the incorporation of precursors. As shown in Table 10, the effect of tetrahydroxyanthraquinone on macromolecular syntheses in *B. brevis* was more or less similar to that of catenarin. Emodin, at low concentrations (1 µg/ml), affected preferentially the incorporation of uracil. The incorporation of leucine was inhibited at higher concentrations (10 µg/ml). Viocristin, 10 µg/ml, inhibited all three macromolecular syntheses.

Thanks are due to Miss A. Archut for conducting this experiment.

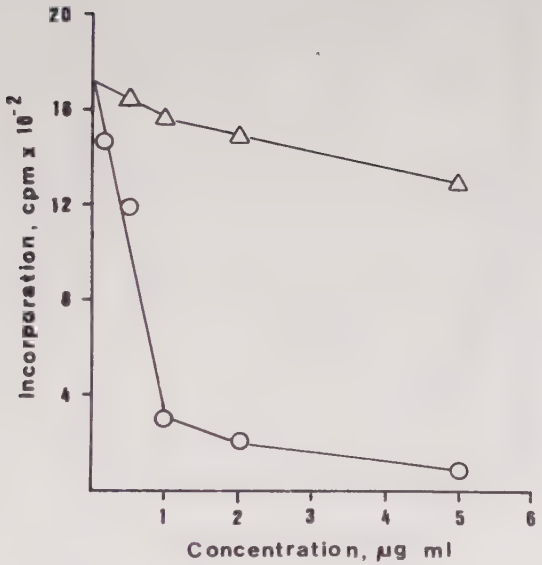


Fig. 12: Effect of catenarin and rubrocristin on the incorporation of (^{14}C) uracil into acid-insoluble fraction in *B. brevis*.

△-△ , rubrocristin

○-○ , catenarin

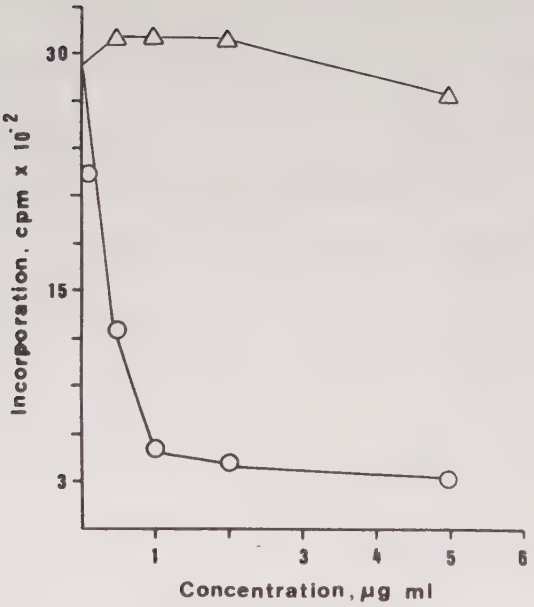


Fig. 13: Effect of catenarin and rubrocristin on the incorporation of (^{14}C) leucine into acid-insoluble fraction in *B. brevis*.

△-△ , rubrocristin

○-○ , catenarin

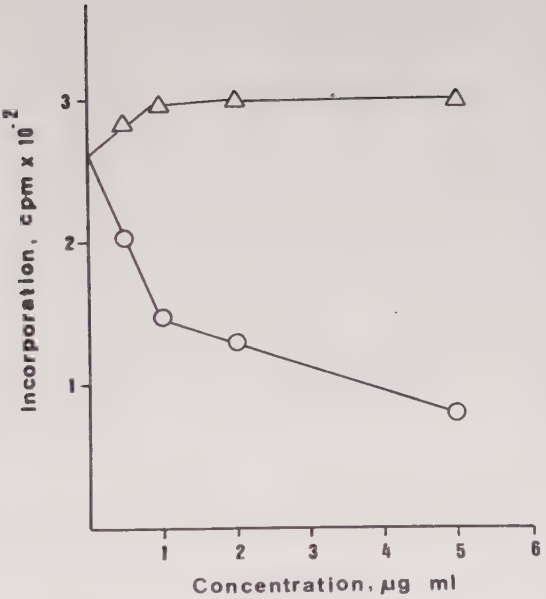


Fig. 14: Effect of catenarin and rubrocristin on the incorporation of (^{14}C) thymidine into acid-insoluble fraction in *B. brevis*.

△-△ , rubrocristin

○-○ , catenarin

Table 10: Effect of polyhydroxyanthraquinones on the incorporation of uracil, leucine and thymidine into TCA-precipitable fraction of cells of *B. brevis*

Compound	(µg/ml)	Incorporation (% of the control) Uracil	Leucine	Thymidine
Tetrahydroxyanthraquinone	1	12	10	58
Tetrahydroxyanthraquinone	10	2	2	22
Catenarin	1	14	16	61
Catenarin	10	2	3	25
Rubrocristin	1	90	105	110
Rubrocristin	10	85	92	106
Emodin	1	12	40	69
Emodin	10	3	12	46
Viocristin	1	96	84	85
Viocristin	10	13	2	2

e. Effect on macromolecular syntheses in ECA cells

Incorporation of leucine, uridine and thymidine into TCA-insoluble material in ECA cells was determined in presence of some anthraquinones. The results calculated as percent of the control are given in Table 11. Catenarin and tetrahydroxyanthraquinone inhibited preferentially the incorporation of uridine. At higher concentrations (10 µg/ml) more than 50 % inhibition in the incorporation of thymidine was also recorded. However, the tested anthraquinones were found to have a slight or no effect on the incorporation of leucine. Emodin caused a lower decrease in the incorporation of uridine while erythroglaucin, physcion and rubrocristin showed no significant inhibitory activity.

Table 11: Effect of polyhydroxyanthraquinones on the incorporation of leucine, uridine and thymidine into TCA-precipitable material in ECA cells.

Compound	(µg/ml)	Incorporation (%) leucine	uridine	thymidine
Erythroglaucin	10	100	82	100
Physcion	10	100	84	101
Rubrocristin	10	102	75	89
Emodin	10	99	40	89
Tetrahydroxyanthraquinone	10	68	6	41
Catenarin	1	91	39	100
Catenarin	2	90	30	72
Catenarin	10	75	7	43
Viocristin	1	100	48	82
Viocristin	2	98	38	73
Viocristin	10*	98	26	67

* the antibiotic was not completely dissolved

f. Inhibition of DNA-dependent RNA polymerase from *E. coli*

From the above results, it can be concluded that catenarin and tetrahydroxyanthraquinone interfere with protein synthesis and/or RNA-synthesis in *B. brevis* and with the latter process in ECA cells. Since the mechanism of protein synthesis is rather complicated it was reasonable to test first the effect of these pigments on the synthesis of RNA *in vitro*.

Data of Table 12 show the influence of some polyhydroxyanthraquinones on the activity of DNA-dependent RNA polymerase from *E. coli* (E.C.2.7.7.6). Catenarin and tetrahydroxyanthraquinone were found to be equally inhibitory, emodin was only slightly inhibitory and the other tested compounds had no effect on the enzyme activity. About 60 % decrease in the enzyme activity was observed at concentrations of 20 µg/ml of catenarin or tetrahydroxyanthraquinone but the same amount of emodin caused only 24 % inhibition. The inhibition of enzyme activity by catenarin was highly dependent on the DNA-concentration in the assay. When the amount of DNA was doubled (40 µg/ml) the effect of catenarin was reduced and when 80 µg/ml DNA were used the effect was completely reversed. From these results an interaction between catenarin and DNA may be expected.

Table 12: Inhibition of DNA-dependent RNA polymerase from *E. coli* by polyhydroxyanthraquinones.

Compound	(µg/ml)	AMP incorporated (% of the control)	
Catenarin	10	65	
Catenarin	20	38	
Catenarin	20	57	2 x DNA
Catenarin	20	108	4 x DNA
Catenarin-6,8-dimethyl ether	10	111	
Emodin	10	84	
Emodin	20	76	
Erythroglaucin	10	115	
Rubrocristin	10	100	
Rubrocristin	20	89	
Physcion	10	112	
Tetrahydroxy-anthraquinone	10	62	
Tetrahydroxy-anthraquinone	20	33	

g. Binding to DNA

Interaction between dyes and DNA can be demonstrated by different methods such as cosedimentation of the dye with DNA in the ultracentrifuge (Kersten and Kersten, 1965), by equilibrium dialysis (Wells and Larson, 1970), by a metachromic shift of the visible absorption spectrum of the dye to longer wavelength in presence of DNA (Kirk, 1960) or by disappearance of the waves of polarographic reduction characteristic for free anthraquinones (Calendi et al., 1965). The spectroscopic method, being simple and convenient, was followed in the present study.

As examples, the spectra of catenarin and rubrocristin, registered immediately after mixing the solutions are presented in Fig. 15 and 16. The compounds studied and the results are listed in Table 13. The presence of DNA resulted immediately in red-shift of the absorption maxima of catenarin and tetrahydroxyanthraquinone solutions. No interaction between rubrocristin (catenarin-8-methyl ether), catenarin-6,8-dimethyl ether or erythroglaucin and DNA was observed even after 24 hours. Physcion showed some binding to DNA noticeable after one day. The addition of DNA to a solution of emodin under the chosen conditions (pH 7.2) gave a blue-shift of 5 nm. The presence of magnesium ions did not change the results obtained with emodin.

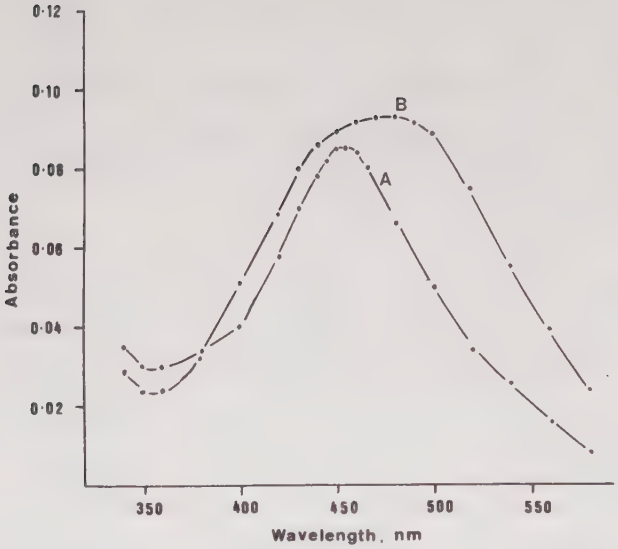


Fig. 15: The effect of DNA on the visible-absorption spectrum of catenarin.

A: 4 mg catenarin in 8 ml ethanol per liter buffer.

B: 4 mg catenarin in 8 ml ethanol + 100 mg DNA per liter buffer.

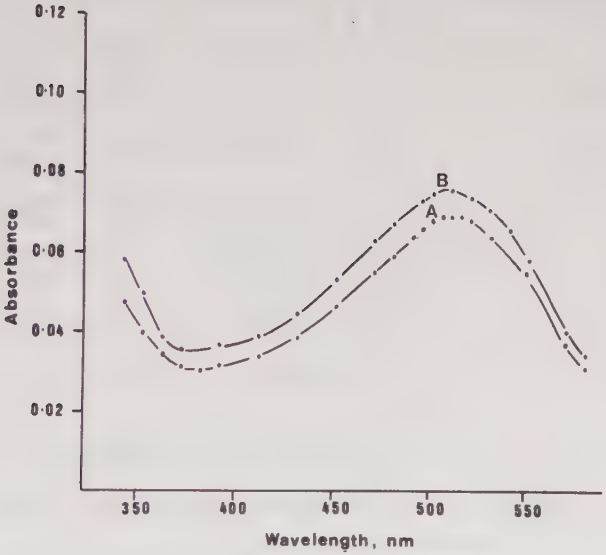


Fig. 16: The effect of DNA on the visible-absorption spectrum of rubrocristin.

A: 4 mg rubrocristin in 8 ml ethanol per liter buffer.

B: 4 mg rubrocristin in 8 ml ethanol + 100 mg DNA per liter buffer.

Table 13: Red-shift of polyhydroxyanthraquinones in the presence of DNA

Compound	λ max. immediately		λ max. after 24 h	
	A	B	A	B
1,4,6,8-Tetrahydroxy-anthraquinone	466	494	464	490
Catenarin	455	475	445	480
Rubrocristin	512	512	515	515
Catenarin-6.8-dimethyl ether	490	490	492	492
Erythroglaucin	450	456	448	448
Physcion	420	430	420	440
Emodin	462	455	475	470

A: anthraquinone B: anthraquinone + DNA

In an attempt to find structure-activity relationship, the ability of a number of anthraquinone derivatives to interact with DNA was compared with their antibiotic activity (Table 14). Some compounds, such as anthragallol, purpurin and emodin did not bind to DNA but they exhibited antibiotic activity. On the other hand, other group of pigments, 1,4-dihydroxy-8-methoxyanthraquinone, physcion and 1,4-dihydroxy-6,8-dimethoxyanthraquinone interacted with DNA but showed no antibiotic activity at concentrations up to 20 µg/disc.

Table 14: Binding of anthraquinone derivatives to DNA and their activity (20 µg/disc) against *B. brevis* 10^6 spores/ml in agar diffusion assay.

Compound	Red-shift	Antibiotic activity
1,2-dihydroxy-A (alizarin)	-	-
1,5-dihydroxy A (anthrarufin)	-	-
1,3,5,7-tetrahydroxy-A	-	-
1,4,8-trihydroxy-6-methoxy-3-methyl-A (erythroglaucin)	-	-
1,4,6-trihydroxy-8-methoxy-3-methyl-A (rubrocristin)	-	-
1,4-dihydroxy-6,8-dimethoxy-3-methyl-A (catenarin dimethylether)	-	-
1,2,3-trihydroxy-A (anthragallol)	-	+
1,2,4-trihydroxy-A (purpurin)	-	+
1,6,8-trihydroxy-3-methyl-A (emodin)	-	+
1,2,3,8-tetrahydroxy-6-methyl-A	-	+
1,2,6,8-tetrahydroxy-3-methyl-A	-	+
1,8-dihydroxy-A (chrysazin)	+	+
1,8-dihydroxy-3-methyl-A (chrysophanic acid)	+	+
1,4,6,8-tetrahydroxy-A	+	+
1,4,6,8-tetrahydroxy-3-methyl-A (catenarin)	+	+
1,4-dihydroxy-8-methoxy-A	+	-
1,8-dihydroxy-6-methoxy-3-methyl-A (physcion)	+	-
1,4-dihydroxy-6,8-dimethoxy-A	+	-

A: anthraquinone

IV. DISCUSSION

1. Cultural conditions and pigment production

No or little effect of the incubation temperature or the starting pH of the medium on growth and on pigment production of *Eurotium cristatum* were observed. The ability of this organism to tolerate a wide range of temperature, being a member of *E. amstelodami* group, has been revealed by Blaser (1974). In addition, species of the *Aspergillus glaucus* series are known to grow within pH values ranging from 4 to 8 (Gould and Raistrick, 1934). pH values above 6.5 were not investigated here to minimize the risk of bacterial contamination.

Maximal production of antibacterially active pigments by *E. cristatum* in submerged cultures was observed between the sixth and eleventh day of incubation. The same result was obtained by Gould and Raistrick (1934), who studied the production of flavoglaucin and auroglaucin by *A. novus* in surface cultures. They related the high pigment production to the abundant cleistothecial perithecia formation during this period. They also observed no additional production of pigments when conidia formation took place (11-13 days).

It is well known that the medium strongly influences the production of secondary metabolites in fungi, as well as in bacteria (Berry, 1975; Bu'Lock, 1975). This should be true for the *A. glaucus* group, in which the development of

cleistothecial stage and the production of characteristic pigments are highly stimulated in media with low nitrogen content and high sugar concentrations, whereas media with high nitrogen content accentuate the condial stage (Raper and Fennell, 1965). The positive effect of glucose (medium 4) on both growth and pigment production has been confirmed by the findings presented here. When small amounts of sodium chloride (2.5 %) were added to malt extract medium (medium 5), the highest content of pigments per gram mycel was obtained. The stimulative effect of 2-5 % sodium chloride on growth and pigment production by *A. umbrosus* was observed by Carbone and Johnson (1964). Moreover, *E. cristatum* showed relatively good growth and pigmentation on malt extract medium. The ability of this organism to grow well and mature normal ascospores quickly on malt extract agar was noted by Raper and Fennell (1965). When the production of pigments by other strains of *A. glaucus* group is considered, it is noticed that the effect of medium varied from one group to the other. The strong effect of medium on pigment production by strains of *A. glaucus* group was noticed before (Gould and Raistrick, 1934; Chollet and Moreau, 1966 and 1967; Anke et al., 1978). More pigments were produced on a "rich" medium by members of group I, while a medium having a high osmotic pressure was favorable for strains of *E. echinulatum* (group II). Species belonging to group III showed varying responses to different media. Three members of this group, *E. montevidensis*, *E. amstelodami* and *E. heterocaryoticum*, produced no pigments under the described conditions. According to Blaser (1974)

E. montevidensis (Talice et Mackinnon) Malloch et Cain is included in *E. amstelodami* Mangin. *E. heterocaryoticum* differs from *E. amstelodami* only in the colour of the conidia. The close relationship of these species is underlined by the obtained results. On the other hand, it should be emphasized that the occurrence and distribution of the pigments, being highly dependent on the medium, can not be considered of taxonomic importance. Blaser (1974) noticed that the pigmentation of the mycelium of species of the *A. glaucus* group was strongly dependent on the cultural conditions and at least quantitative variations could be observed from one strain to the other. The quantitative variation of the produced pigments by one and the same strain (*E. cristatum*) in different media has been demonstrated in the present study.

2. Antimicrobial activity of catenarin and other anthraquinone derivatives

Catenarin was found to be the most active compound among the pigments isolated from *E. cristatum*. Tetrahydroxyanthraquinone showed a slightly higher activity in the agar diffusion test which was due to its higher diffusion rate. In liquid medium, in the serial dilution assay, both compounds were equally active. Moreover, their effects on growth of *B. brevis* were exactly the same in complex and minimal media. These results indicate that the methyl group in position 3 does not affect the antibiotic activity. However, methylation of the hydroxyl group in position 6 or

8 leads to antibiotically inactive compounds: erythroglaucin and rubrocristin were not inhibitory at concentrations up to 20 and 25 µg/ml towards *B. brevis*. The same was observed with emodin and its monomethyl ether (physcion): physcion was inactive, whereas emodin showed some antibiotic activity. Podojil and coworkers (1978) isolated physcion and erythroglaucin from mycelia of *E. repens* and reported that physcion exhibited antibacterial activity in the agar diffusion assay. The high amount (125 µg/disc) used by these authors includes the possibility of the presence of antibacterially active impurities. Emodin has been shown to be toxic to ringworm fungi (*in vitro*) while physcion was inactive (Agarwal and coworkers, 1976). Emodin was also found to inhibit the growth of *B. megaterium* (Buckelew et al., 1972) and tubercle bacilli (Ranganatha Rao and Seshadri, 1955).

Viocristin, a derivative of 1,4-anthraquinone, which so far has not been described as a natural product, inhibited the growth of *B. brevis* in solid and liquid media. Its inhibitory effect was higher in the serial dilution assay than that in the agar diffusion assay which indicates poor diffusion in the agar medium.

3. Inhibition of macromolecular syntheses

Catenarin and tetrahydroxyanthraquinone blocked almost completely the incorporation of labeled precursors (leucine and uracil) into protein and RNA in cells of *B. brevis* at 1 µg/ml. DNA synthesis was also affected but only at

higher concentrations. In case of emodin, the biosynthesis of RNA was preferentially inhibited at the low concentration (1 µg/ml) and with a ten-fold higher concentration the synthesis of protein could be affected. This is rather interesting since it indicates different effects of these structurally related compounds on bacteria.

Viocristin inhibited all three macromolecular syntheses. Being a para-benzoquinone derivative, this compound may react with SH-groups resulting in inhibition of various enzyme reactions.

In cells of the ascitic form of Ehrlich carcinoma, catenarin and tetrahydroxyanthraquinone inhibited preferentially RNA-synthesis while DNA-synthesis was only moderately affected at higher concentrations. No inhibitory effect on protein-synthesis was observed. The inhibitory effect of these two compounds, and emodin, towards *B. brevis* was much higher than in ECA cells. Again rubrocristin, erythroglaucin and physcion showed no significant activity. Podojil et al. (1978) reported physcion to be cytotoxic to Hela cells at very low cencentrations (0.1 µg/ml) and emodin was found to be toxic to 1-day old cockerels (Wells et al., 1975) and to the cells of protozoon *Tetrahymena pyriformis* (Ueno and Saheki, 1968). Unfortunately erythroglaucin or other anthraquinone derivatives were not tested.

4. Inhibition of DNA-dependent RNA polymerase from *E. coli* and binding to DNA

Catenarin and tetrahydroxyanthraquinone inhibited significantly the polymerization of nucleotides into RNA *in vitro*. These two compounds, which have the highest antibacterial activity interact immediately with DNA. Rubrocristin, having no antimicrobial activity, did not affect RNA synthesis and gave no red-shift in the presence of DNA. Emodin showed a weak inhibitory effect on RNA polymerase and no significant binding to DNA even in the presence of magnesium ions. The presence of magnesium ions was found to be essential for complex formation between DNA and some antibiotics or pigments such as chromomycin or luteoskyrin (Behr and Hartmann, 1965; Ward et al., 1965; Ueno et al., 1968). The binding of emodin to DNA was found to depend on the pH of the buffer solution; only at pH 4.3 an interaction between emodin and DNA was observed (Swanbeck and Zetterberg, 1971).

5. Structure activity relationship

It is surprising that two closely related compounds like catenarin and emodin, the former having one more hydroxyl group in position 4 than the latter, show diverse biological effects in bacteria and towards DNA and RNA polymerase *in vitro*. Therefore, the number and positions of different substituents should be considered as an important factor which could affect the affinity of the pigment to DNA and thus its antibiotic activity. When nineteen anthraquinone derivatives were tested, two groups of compounds could be

recognized in addition to those which were able to bind to DNA and to inhibit bacterial growth. One group included pigments, having a methylated hydroxyl group in position 6 and/or 8, that interacted with the nucleic acid but did not show antibiotic activity. This may be due to transport difficulties, which prevent the compounds to reach their site of action within the cell. The possibility of inactivation through chemical alteration may also be considered. The other group of anthraquinones, that have three or more hydroxyl groups one of which is in position 2 (except emodin), did not bind to DNA at pH 7.2 but were antibacterially active. Two members of this group, emodin and purpurin, were found to bind to DNA at pH 4.3 but not at pH 6.8 (Swanbeck and Zetterberg, 1971). Purpurin and anthragallol were reported to induce direct mutations of the frameshift type in *Salmonella typhimurium* (Brown and Brown, 1976). They were also reported to be toxic to earthworms (Lagrange, 1946). Purpurin was found to exhibit antitumor activity when injected into tumor-bearing mice (Powell, 1944).

In order to understand the stereochemical criteria for binding of the tested anthraquinones to DNA, the structures and the obtained red-shift (if any) are given in Table 15. From this table the following could be concluded:

1. Anthraquinone derivatives, that give a red-shift, have at least two OH-substitutions at positions 1 and 4 or 1 and 8.
2. Substitution with a bulky group (e.g. OCH_3) decreases the ability of the substance to bind to DNA.

3. The presence of an OH-group in position 2 prevents complex formation.
4. Anthraquinone derivatives, having five substituions one or more of which is a bulky group, give no red-shift with DNA.

Berg and Eckardt (1970) found no significant influence of the number and positions of the chromophore hydroxyl groups on the binding ability of anthracycline antibiotics to DNA. However, Swanbeck and Zetterberg (1971) noticed that substitution with small polar groups, such as hydroxy- and methoxy-groups, increases the degree of binding to DNA but if the substitution makes the compound a negative ion at certain pH, no binding occures. This is the case for hydroxy substitutions in the ß-positions (positions 2,3,6 or 7). These findings are partially in agreement with the above mentioned conclusions: emodin has three hydroxyl groups two of them, in position 1 and 8, promote binding to DNA while the third one hinders this binding. In physcion the 6-hydroxyl group, which hinders binding to the nucleic acid, is methylated and thus some binding was observed. Compared to emodin, catenarin possesses a forth hydroxyl group in position 4 which promotes binding. As a result of three promoting and one hindering groups this compound binds immediately to DNA.

Table 15: Effect of number and positions of substituents on the binding of anthraquinones to DNA.

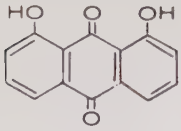
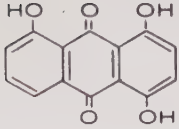
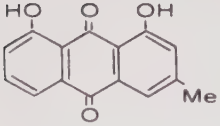
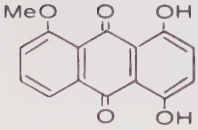
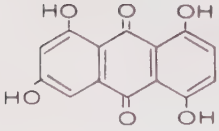
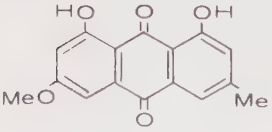
Structure	Name	Red-shift nm
	CHRYSAZIN 1,8-DIHYDROXY- ANTHRAQUINONE	30-40
	1,4,8-TRIHYDROXY- ANTHRAQUINONE	35
	CHRYSOPHANIC ACID 1,8-DIHYDROXY-3-METHYL- ANTHRAQUINONE	30-40
	1,4-DIHYDROXY-8-METHOXY- ANTHRAQUINONE	22-28
	1,4,6,8-TETRAHYDROXY- ANTHRAQUINONE	25-30
	PHYSCION 1,8-DIHYDROXY-6-METHOXY- 3-METHYLANTRAQUINONE	10-20

Table 15, cont.

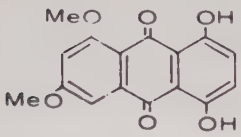
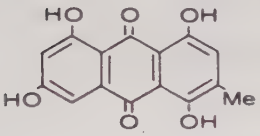
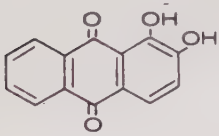
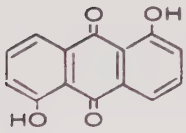
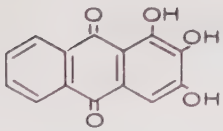
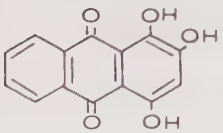
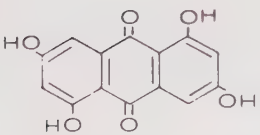
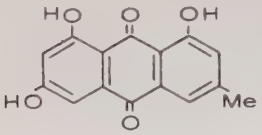
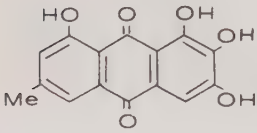
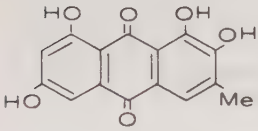
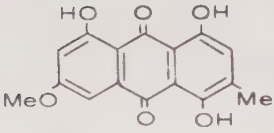
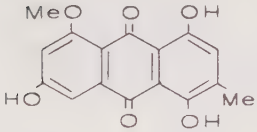
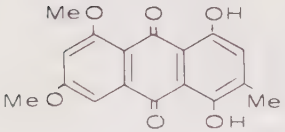
	1,4-DIHYDROXY-6,8-DIMETHOXYANTHRAQUINONE	15-18
	CATENARIN 1,4,6,8-TETRAHYDROXY-3-METHYLANTHRAQUINONE	20-35
	ALIZARIN 1,2-DIHYDROXYANTHRAQUINONE	-
	ANTHRARUFIN 1,5-DIHYDROXYANTHRAQUINONE	-
	ANTHRAGALLOL 1,2,3-TRIHYDROXY-ANTHRAQUINONE	-
	PURPURIN 1,2,4-TRIHYDROXY-ANTHRAQUINONE	-
	1,3,5,7-TETRAHYDROXY-ANTHRAQUINONE	-

Table 15, cont.

	EMODIN 1,6,8-TRIHYDROXY-3- METHYLANTHRAQUINONE	-
	1,2,3,8-TETRAHYDROXY-6- METHYLANTHRAQUINONE	-
	1,2,6,8-TETRAHYDROXY-3- METHYLANTHRAQUINONE	-
	ERYTHROGLAUCIN 1,4,8-TRIHYDROXY-6-METH- OXY-3-METHYLANTHRAQUINONE	-
	RUBROCRISTIN 1,4,6-TRIHYDROXY-8-METH- OXY-3-METHYLANTHRAQUINONE	-
	CATENARIN-6,8-DIMETHYLETHER 1,4-DIHYDROXY-6,8-DIMETH- OXY-3-METHYLANTHRAQUINONE	-

6. Mode of action of catenarin

From the preceding data and discussion, it is obvious that catenarin and tetrahydroxyanthraquinone have essentially identical mechanism of action; both compounds form complexes with DNA *in vitro*. According to Lerman (1964) and Bradley and Wolf (1959) two binding types of dyes with DNA have been postulated; the "intercalating type", the dye molecule being inserted between the levels of base pairs of DNA double helix, as in the case of daunomycin (Pigram et al., 1972), and the "stacking type", the positively charged dye molecule being bound to the periphery of the double helix by electrostatic attraction of the negatively charged phosphate groups of the DNA, as in the case of ethidium bromide at high salt concentrations (Aktipis and Kindelis, 1973). Regarding the case of anthraquinone molecules, which possess no positive charges at neutral pH, the first type of binding seems to be an attractive hypothesis. However, further studies on the formed complexes by other methods, which is beyond the scope of this work, are necessary to discriminate the two modes of binding and to give more understanding of the complex formation between anthraquinone derivatives and DNA.

Antibiotics which interact with DNA interfere with the DNA dependent processes. They may prevent either replication of DNA, as phleomycin (Falaschi and Kornberg, 1964), or DNA-dependent RNA synthesis (transcription), like actinomycin (Goldberg et al., 1962; Hartmann and Coy, 1962) or both

processes as noticed with anthracyclines (Koschel et al., 1966).

From the results obtained in this study it seems that the biological action of catenarin depends on its association with DNA. It is thus likely that the presence of catenarin hinders the attachment of RNA polymerase to the DNA template resulting in a decreased number of initiations of new RNA molecules. Evidence for this hypothesis is the inhibition of DNA-dependent RNA polymerase from *E. coli in vitro*, and the reversibility of this inhibition in presence of high concentrations of DNA. Moreover, rubrocristin (catenarin-8-methyl ether) failed to bind to DNA and could affect neither the synthesis of RNA *in vitro* nor any of the tested biological processes.

The inhibition of RNA synthesis results in cessation of protein synthesis, especially in cells with high turn over rate of mRNA. Therefore, the inhibition of protein synthesis by catenarin might be a secondary effect.

Catenarin, at high concentrations, inhibited DNA-synthesis to some extent. The same phenomenon was observed with actinomycin; this was dued to the greater stability of the double helix, brought about by an increased number of drug molecules bound to DNA (Franklin and Snow, 1975). This explanation may be true for catenarin too but further investigations on the effect of catenarin on DNA-dependent DNA polymerase are needed in order to evaluate its validity.

V. SUMMARY

The production of pigments by strains of the *Aspergillus glaucus* group was investigated. Many members of the group were able to produce a number of polyhydroxyanthraquinone derivatives but *Eurotium cristatum* was found to yield the highest amounts. These pigments were therefore isolated from *E. cristatum* and identified as catenarin, erythroglaucin, physcion, physcion-9-anthrone, rubrocristin and viocristin. The last compound does not belong to the 9,10-anthraquinone series but to 1,4-anthraquinones. So far rubrocristin and viocristin have not been reported among naturally occurring quinones.

The antimicrobial properties of all substances together with emodin and a synthetically related compound, 1,4,6,8-tetrahydroxyanthraquinone, were examined. Gram-positive bacteria were the most sensitive organisms and catenarin was the most active naturally occurring substance. Tetrahydroxyanthraquinone showed the same antibiotic activity as catenarin, emodin and viocristin were less active than catenarin, whereas erythroglaucin, physcion and rubrocristin showed no antimicrobial activity.

In exponentially growing cells of *Bacillus brevis* catenarin and emodin inhibited preferentially the incorporation of uracil and leucine. At higher concentrations the incorporation of thymidine into acid-precipitable fractions of cells was also affected. In the presence of viocristin all three macromolecular syntheses came to a halt.

In Ehrlich ascites carcinoma cells catenarin, emodin and viocristin inhibited preferentially the incorporation of uridine. The incorporation of thymidine was affected to a lesser extent, while that of leucine was hardly affected.

In vitro inhibition of DNA-dependent RNA polymerase from *Escherichia coli* by catenarin and to a lesser extent by emodin was also observed, whereas rubrocristin, physcion and erythroglaucin were not active.

Binding of the isolated pigments, as well as a number of synthetic anthraquinones, to DNA was measured. Catenarin interacted immediately with DNA, while rubrocristin, erythroglaucin and emodin did not give a red-shift in the presence of the nucleic acid. The effect of the number and positions of substituents on the affinity of anthraquinone derivatives to DNA is discussed.

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